09759841 12-08-98

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C07K 1/00, 14/00, 17/00, A61K 39/00, 39/38, 39/21, 39/12

A1

(11) International Publication Number: **WO 98/15569**

(43) International Publication Date: 16 April 1998 (16.04.98)

(21) International Application Number: PCT/US97/18397

(22) International Filing Date: 8 October 1997 (08.10.97)

(30) Priority Data:
60/027,931 9 October 1996 (09.10.96) US

(71) Applicants *(for all designated States except US)*: DANA-FARBER CANCER INSTITUTE [US/US]; 44 Binney Street, Boston, MA 02115 (US). LEUKOSITE INCORPORATED [US/US]; 215 First Street, Cambridge, MA 02142 (US). CHILDREN'S MEDICAL CENTER CORPORATION [US/US]; 300 Longwood Avenue, Boston, MA 02115 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: SODROSKI, Joseph [US/US]; 10 Ashland Place, Medford, MA 02155 (US). NEWMAN, Walter [US/US]; 3 Durham Street #3, Boston, MA 02115 (US). WU, Lijun [US/US]; 139 Oak Street, Reading, MA 01867 (US). GERARD, Norma [US/US]; 117 Walpole Street, Dover, MA 02030 (US). GERARD, Craig [US/US]; 117 Walpole Street, Dover, MA 02030 (US).

(74) Agents: EISENSTEIN, Ronald, I. et al.; Dike, Bronstein, Roberts & Cushman, LLP, 130 Water Street, Boston, MA 02109 (US).

(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: GP120 POLYPEPTIDES HAVING CONFORMATIONAL DISCONTINUOUS CHEMOKINE RECEPTOR BINDING SITES AND METHODS OF INHIBITING HIV INFECTION

(57) Abstract

A gp120 conformational binding site that is formed by the binding of gp120 and CD4 which permits binding of the complex to the chemokine receptors is disclosed. Binding assays which permit the ready screening for molecules which affect the binding of gp120 and the chemokine are taught as well as specific targets for affecting the binding.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

GP120 POLYPEPTIDES HAVING CONFORMATIONAL DISCONTINUOUS CHEMOKINE RECEPTOR BINDING SITES AND METHODS OF INHIBITING HIV INFECTION

The present invention was funded in part by grants from the United States Government and it has certain rights to the inventions described herein.

The present invention claims priority from U.S. Provisional applications 60/027,931; the content of that application is incorporated herein by reference.

The present invention is directed to gp120 polypeptides having a conformational discontinous chemokine receptor binding site and methods for inhibiting HIV infectivity.

Human immunodeficiency viruses type 1 and type 2 (HIV-1 and HIV-2) are the etiologic agents of acquired immunodeficiency syndrome (AIDS) in humans (Barre-Sinoussi *et al.*, 1984). AIDS results from the depletion of CD4-positive T lymphocytes in HIV-infected individuals (Fauci *et al.*, 1984).

HIV-1 infects T lymphocytes, monocytes/macrophage, dendritic cells and, in the central nervous system, microglia (Gartner *et al.*, 1986; Koenig *et al.*, 1986; Pope *et al.*, 1994; Weissman *et al.*, 1995). All of these cells express the CD4 glycoprotein, which serves as the receptor for HIV-1 and HIV-2 (Dalgleish *et al.*, 1984; Klatzman *et al.*, 1984; Maddon *et al.*, 1986). Efficient entry of HIV-1 into target cells is dependent upon binding of the viral exterior envelope glycoprotein, gp120, to the CD4-amino-terminal domain (McDougal *et al.*, 1986; Helseth *et al.*, 1990). After virus binding, the HIV-1 envelope glycoproteins mediate the fusion of viral and host cell membranes to complete the entry process (Kowalski *et al.*, 1987; Stein *et al.*, 1987; Helseth *et al.*, 1990). Membrane fusion directed by HIV-1 envelope glycoproteins expressed on the infected cell surface leads to fusion with uninfected CD4-positive cells, resulting in syncytia (Lifson *et al.*, 1986; Sodroski *et al.*, 1986).

Host cell factors in addition to CD4 appear necessary for effective HIV-1 envelope glycoprotein-mediated membrane fusion. Some human and

animal cells have been shown to be resistant to HIV-1 infection and syncytium formation even when human CD4 was expressed on the cell surface (Maddon *et al.*, 1986; Ashorn *et al.*, 1990; Chesebro *et al.*, 1990; McKnight *et al.*, 1994). Experiments with somatic cell hybrids suggested the possibility that a positive factor expressed in cells susceptible to syncytium formation could complement the block to fusion in resistant cell types (Clapham *et al.*, 1991; Dragic *et al.*, 1992; Broder *et al.*, 1993). HIV-1 variants exhibiting distinct differences in the ability to fuse with and to enter particular subsets of CD4-positive cells have been identified (Broder and Berger, 1995).

Discovery of factors that enhance HIV entry is important because it provides new areas for attacking the virus, new diagnostic and prognostic screens and can permit monitoring the susceptibility to HIV infection and/or development of AIDS.

All primary clinical HIV-1 isolates, defined as viruses that have not been passaged on immortalized cell lines, replicate in primary monocytes/macrophages and in primary T lymphocytes. Two groups of primary HIV-1 isolates have been defined, based on replication rate in peripheral blood mononuclear cells (PBMC) and the ability to infect and induce the formation of syncytia in immortalized CD4-positive cell lines (Asjo *et al.*, 1986; Cheng-Mayer *et al.*, 1988; Fenyo *et al.*, 1988; Tersmette *et al.*, 1988).

Most primary HIV-1 viruses that initiate human infection and that persist throughout the course of infection replicate to low levels in PBMC and do not replicate in immortalized T cell lines (Asjo *et al.*, 1986; Schuitemaker *et al.*, 1991; Schuitemaker *et al.*, 1992; Connor *et al.*, 1993, 1994a,b). These viruses are referred to herein as macrophage-tropic primary isolates (sometimes referred to as "M"). In some HIV-1-infected individuals, viruses that replicate to higher levels in PBMC and that can infect and induce the formation of syncytia in immortalized CD4-positive cell lines emerge late in the course of infection (Asjo *et al.*, 1986; Schuitemaker *et al.*, 1992; Connor *et al.*, 1993, 1994a,b). These viruses will be referred to herein as T cell line-tropic primary viruses (sometimes referred to as "T") The T cell line-tropic primary viruses, by virtue of their ability to replicate on some immortalized cell lines, serve as precursors to the laboratory-adapted

isolates, which have been extensively passaged on such cell lines. Laboratory adaptation, however, results in a loss of the ability of HIV-1 to replicate in primary monocyte/macrophage cultures (Schuitemaker *et al.*, 1991; Chesebro *et al.*, 1991; Westervelt *et al.*, 1992; Valentin *et al.*, 1994). Thus, while all HIV-1 isolates replicate on primary T lymphocytes, three groups of virus variants can be defined based on the ability to replicate in primary monocyte/macrophages or in immortalized T cell lines: (1) macrophage-tropic primary viruses that cannot infect T cell lines; (2) laboratory-adapted viruses that cannot infect primary monocytes/macrophages; and (3) T cell line-tropic primary viruses that exhibit dual-tropism for these cell types.

Changes in the viral envelope glycoproteins, in particular in the third variable (V3) region of the gp120 exterior envelope glycoprotein, determine tropism-related phenotypes (Cheng-Mayer *et al.*, 1990; O'Brien *et al.*, 1990; Hwang *et al.*, Westervelt *et al.*, 1992; Chesebro *et al.*, 1992; Willey *et al.*, 1994). Amino acid changes in the V3 region (Helseth *et al.*, 1990; Freed *et al.*, 1991; Ivanoff *et al.*, 1991; Bergeron *et al.*, 1992; Grimaila *et al.*, 1992; Page *et al.*, 1992; Travis *et al.*, 1992) and the binding of antibodies to this domain (Putney *et al.*, 1986; Goudsmit *et al.*, 1988; Linsley *et al.*, 1988; Rusche *et al.*, 1988; Skinner *et al.*, Javeherian *et al.*, 1989) have been shown to disrupt a virus entry process other than CD4 binding. The dependence of the phenotype resulting from V3 structural variation on the particular target cell suggested that the V3 region, which contains a surface-exposed, disulfide-linked loop (Leonard *et al.*, 1990; Moore *et al.*, 1994), might act in conjunction with target cell moieties to determine the efficiency of membrane fusion events.

Infection of macrophage-tropic primary HIV-1 isolates, but not that of a laboratory-adapted isolate, has been shown to be inhibited by the β-chemokines RANTES, MIP-1α and MIP-1β (Cocchi *et al.*, 1995). High endogenous expression of these β-chemokines has been suggested to account for the *in vitro* resistance to HIV-1 infection of CD4-positive T cells from uninfected individuals with multiple sexual exposures to seropositive partners (Paxton *et al.*, 1996). This resistance was only seen for macrophage-tropic and not T cell line-tropic viruses and was influenced by the structure of the third variable (V3) gp120 region of the infecting virus.

Recently, an "orphan" G protein-coupled seven transmembrane segment receptor, variously called *HUMSTR, LCR-1* or *LESTR* (now referred to as CXCR4) (Federsppiel *et al.*, 1993; Jazin *et al.*, 1993; Loetscher *et al.*, 1994) has been shown to allow a range of non-human, CD4-expressing cells to support infection and cell fusion mediated by laboratory-adapted HIV-1 envelope glycoproteins (Feng *et al.*, 1996). Antibodies to *HUMSTR* blocked cell fusion and infection by laboratory-adapted HIV-1 isolates but not by macrophage-tropic primary viruses (Feng *et al.*, 1996). While its natural ligand is currently unknown, *HUMSTSR* exhibits sequence similarity to the receptor for interleukin-8, an alpha (CXC) chemokine) (Probst *et al.*, 1992). The available data raised the possibility that at least one other molecule in addition to CD4 and distinct from CXCR4 facilitates the entry of primary, macrophage-tropic HIV-1 isolates, and that this molecule might be influenced by interaction with β-chemokines. Discovery of what the molecules are is desirable because it will help to modulate HIV cellular entry and develop more effective screening assays for inhibitors.

Chemokines are a family of structurally related peptides that recruit leukocytes to inflammatory lesions, induce release of granule contents from granulocytes, regulate integrin avidity, and in general exhibit proinflammatory properties. The α chemokines, or CXC chemokines, primarily activate neutrophils, while the β chemokines or CC chemokines, generally activate monocytes, lymphocytes, basophils and eosinophils (Baggiolini *et al.*, 1994; Schall and Bacon, 1994). Receptors to these chemokines belong to the G protein-coupled receptor family. The large family of G protein-coupled receptors responds to chemoattractants, neurotransmitters, peptide hormones, light and odorants. Amino acid identity among receptors that bind functionally related ligands ranges from 20-80% (Probst *et al.*, 1992; Gerard and Gerard, 1994). Seven transmembrane receptors that transduce their signals through heterotrimeric G proteins are used by leukocytes to respond to chemokines (Horuk *et al.*, 1994). There are a number of closely related molecules in the CC chemokine receptor family but only six of these have been characterized in ligand binding assays. These are designated CCR1, CCR2A, CCR2B, CCR3, CCR4 and CCR5. (They have previously been referred to is C-C CKR-1, -2A, -2B, 3, -4 and -5).

SUMMARY OF THE INVENTION

We have also discovered that a gp120 conformational binding site is formed by the binding of gp120 and CD4 which permits binding of the complex to the chemokine receptors.

We have also discovered binding assays which permit the ready screening for molecules which affect the binding of gp120 and the chemokine.

We have also discovered specific targets for affecting the binding.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-1D show CAT activity in transfected HeLa cells exposed to recombinant HIV-1 viruses. HeLa cells expressing human CD4 only are shown in Fig. 1A, CD4 and CCR1F are shown Fig. 1B, CD4 and CCR3 are shown in Fig. 1C and CD4 and CCR5 are shown in Fig. 1D. The cells were exposed to recombinant viruses containing either no envelope glycoproteins (None) or envelope glycoproteins of the ADA, YU2, Br20-4 or HXBc2 isolates. The results of the CAT assay performed on the HeLa cell lysates are shown.

Figure 2 shows the effect of eotaxin on CCR3-mediated enhancement of YU2 recombinant virus. HeLa-CD4 cells transfected with plasmids expressing CD2, CCR1F, CCR3, CCR3F or CCR5 were incubated for one hour at 37°C with increasing amounts of eotaxin. Recombinant HIV-1 viruses containing the envelope glycoprotein of the YU2 macrophage-tropic primary isolate were added to the cells. CAT activity in the cell lysates was assessed 72 hours later.

Figures 3A-3D show the effect of CCR3, CCR5 and HUMSTSR expression on HIV-1 infection of Cf2Th canine thymocytes. Cf2Th canine thymocytes expressing human CD4 only (Fig 3A), CD4 and CCR3 (Fig 3B), CD4 and CCR5 (Fig 3C), or CD4 and HUMSTSR (Fig 3D) were infected with recombinant viruses containing the indicated envelope glycoproteins. The CAT assay results are shown. The results of a single experiment are shown. Comparable results were obtained in a repeat experiment.

Figures 4A-D show the effect of chemokine receptor expression on HIV-1 envelope glycoprotein-directed syncytium formation. HeLa cells expressing either no envelope glycoprotein (None) or the ADA, YU2, HXBc2 (ADA-V3), and HXBc2) (YU2-V3) envelope glycoproteins were cocultivated with HeLa-CD4 expressing CCR1, CCR3 (Fig 4B) or CCR5 (Figs. 4C and 4D).

In one set of experiments, 2 μg/ml of the OKT4a antibody (Ortho Pharmaceuticals, Inc.) (Fig. 4D) was added at the beginning of the cocultivation. After 12 hours, the syncytia in the wells were counted. The results of a single experiment are shown. The experiment was repeated with comparable results.

Fig. 5 shows the effect of eotaxin on CCR3-mediated enhancement of a recombinant virus containing amphotropic murine leukemia virus (A-MuLV). HeLa-CD4 cells which were transfected with plasmids expressing CD2, CCR1F or CCR3 under the same conditions as described in Figure 2. A chimeric HIV-1 virus containing the A-MuLV envelope glycoproteins were added to the cells. CAT activity in the cell lysate was assayed 72 hours later.

Figures 6A-C show the results of RT-PCR analysis of primary human brain cultures. Figure 6A shows detection of CCR5, CXCR4 and CCR3 transcripts in primary brain culture. Figure 6B shows detection of CCR3 in microglia but not other brain cell types by double immunofluoresence staining. Figure 6C shows detection of CCR3 in microglia.

Figure 7 shows identification of HIV-1 infected cells (YU2 or ADA infected) using GFP fluorescence in combination with cell specific markers as indicated.

Figure 8A-D show the efficiency of early phase virus replication as determined by measuring luciferase activity in primary brain culture.

In Figure 8A □ = no treatment, ■ = MIP-1β, /// = Eotaxin, □ = SDF-1, □ = MCP-1. In Fig. 8B, the legend is the same, in addition, □ = anti-CCR3 (7B11) ■ (last solid box) = RANTES, □ = Eotaxin and MIP-1β. In Fig. 8C the legend is the same, in this case, the only solid box represents RANTES. In Figure 8D, CAT activity is measured.

Figures 9A-E show inhibition of MIP-1α and MIP-1β binding to chemokine receptor-expressing cells by gp120 glycoproteins in the absence and presence of sCD4. Figure 9A shows the gp120 glycoproteins used in the study, indicating the conserved (C1-C5) and variable (V1-V5) regions of the native gp120 glycoproteins included in each of the proteins. The YU2 derivatives are chimeric molecules containing YU2 sequences (shown in white) sufficient for CCR5 utilization [Choe, H., et al., Cell 85:1135-1148 (1996)], as well as sequences (shown in black) derived from the HXBc2

gp120 glycoprotein. Figure 9B shows the gp120 glycoprotein variants tested for ability to inhibit MIP-1α binding to CCR5F-L1.2 cells. In some experiments, sCD4 was included (at 100 nM final concentration unless otherwise noted). Figure 9C shows the effect of different doses of gp120 variants on MIP-1α binding to CCR5F-L1.2 cells, in the absence (broken lines and open symbols) or presence (solid lines and closed symbols) of 100 nM sCD4. Results are shown for the JR-FL (▽,▼), BAL (△,▲), YU2ΔC1ΔV1/2ΔC5 (○,●), HXBc2 (◊), HXBc2ΔC1ΔV1/2/3ΔC5 (□) and YU2ΔC1ΔV1/2/3ΔC5 (✧)glycoproteins. Figure 9D shows the gp120 variants tested, in the presence of 100 nM sCD4, for ability to inhibit MIP-1α binding to CCR1-L1.2 cells. Figure 9E shows gp120 variants tested for the ability to inhibit MIP-1β binding to CCR5F-L1.2 cells in the absence and presence of 100 nM sCD4.

Figures 10A-C show comparison of two-domain and four-domain soluble CD4 proteins for ability to inhibit MIP-1α and MIP-1β binding in the absence and presence of gp120 glycoproteins. Figure 10A shows the ability of D1D2 sCD4 (△,▲), sCD4 (○,●), and soluble VCAM (□,■) to inhibit MIP-1α binding to CCR5F-L1.2 cells in the absence (open figures) and presence (closed figures) of 50 nM BAL gp120. Figure 10B shows the ability of D1D2 sCD4 (△,▲) and sCD4 (○,●) to inhibit MIP-1β binding to CCR5F-L1.2 cells in the absence (open figures) and presence (closed figures) of 50 nM YU2ΔC1ΔV1/2ΔC5 glycoprotein. Figure 10C shows the D1D2 sCD4 (△) and sCD4 (○) proteins ability to inhibit MIP-1α binding to CC41-L1.2 cells at the indicated concentrations, in the absence of gp120 glycoproteins.

Figure 11 shows the effects of monoclonal antibodies on the inhibition of MIP-1α binding to CCR5-expressing cells by gp120-sCD4 mixtures. Monoclonal antibodies (final concentration 500 nM) directed against gp120 (black shading), against CD4 (white shading) or against hybrid gp120-CD4 epitopes (grey shading) were tested for the ability to affect the inhibition of MIP-1α binding to CCR5F-L1.2 cells by a mixture of the JR-FL gp120 glycoprotein (50 nM final concentration) and sCD4 (100 nM final concentration). The inhibition of MIP-1α binding to CCR5F-L1.2 cells by the JR-FL gp120-sCD4 mixture in the absence of added antibody is also shown (hatched bar).

Figures 12A and B show binding of a radiolabeled macrophage-tropic primary virus gp120 derivative to CCR5-expressing cells. Figure 12A shows binding in the presence of 100 nM sCD4 of iodinated YU2ΔC1ΔV1/2ΔC5 protein to CCR5F-L1.2 cells in the presence of increasing concentrations of either rYU2ΔC1ΔV1/2ΔC5 protein (○) or HXBc2ΔC1ΔC5 protein (□). Figure 12B shows binding in the presence of 100 nM sCD4 and 100 nM HXBc2ΔC1ΔC5 protein, of iodinated YU2ΔC1ΔV1/2ΔC5 protein to CCRF-L1.2 cells in the presence of the indicated concentrations of YU2ΔC1ΔV1/2ΔC5 protein (○), MIP-1α (◊), MIP-1β (□), RANTES (Δ) or YU2ΔC1ΔV1/2/3ΔC5 protein (X).

Figure 13 illustrates the results from a typical experiment for gp120/sCD4/CCR-binding using different amounts of membranes. The signal-to-noise ration (the ratio of total binding vs. non-specific binding) is shown on the top of each membrane concentration.

Figure 14 shows a Scatchard analysis using 5 μg membranes. Unlabeled JRFL-gp120 was added with increasing concentrations and the data analyzed by Scatchard analysis. Binding affinity is kd=0.7 nM. βmax=1.6 pmol/mg membrane.

Figure 15 shows that as a positive control, anti-CCR5 mAb 2D7 can efficiently inhibit the binding of ^{125}I-gp 120/sCD4 to CCR5.

Figure 16 is a comparison of the amino-terminal sequence of gpr15, gpr1, rCCR5 and CCR5 with the three conserved tyrosines (Y) shown in bold and reasonably conserved residues underlined. (MDPEETSVYLDYYYATSPN (SEQ ID NO:1); MEDLEETLFEEFENYSYDLDYYSLESD (SEQ ID NO:2); MDYQVSSPTYDIDYYTSEPC (SEQ ID NO:3); and MDYQVSSPIYDINYYTSEPC (SEQ ID NO:4)).

DETAILED DESCRIPTION OF THE INVENTION

β-chemokine receptors are the receptors that bind β-chemokines. β-chemokines are a family of 8-10 kD secreted proteins. These proteins are characterized as β-chemokines based on the absence of an intervening amino acid in the first of two conserved cysteine pairs (CC) as opposed to the α-chemokines that have an intervening amino acid in the first conserved cysteine pair (CXC). The chemokines include macrophage inflammatory protein (MIP-1α and MIP-1β), RANTES (regulated on activation T expressed

and secreted), monocyte chemotactic protein (MCP-1, MCP-2, MCP-3) and eotaxin. The class of surface proteins that bind certain of these chemokines have been identified and belong to the G-protein -coupled seven transmembrane segment receptor family. The chemokine receptors (sometimes referred to as CXR- or CCR-) are characterized based on the specific chemokines they bind to. For example, CCR1 for example binds chemokines MIP-1α and RANTES with high affinity. CCR2A and CCR2B for example bind both MCP-1 and MCP-3. CCR3 for example binds chemokines such as eotaxin, RANTES, and MCP-3 with high affinity. CCR4 for example binds MIP-1α, RANTES and MCP-1. CCR5 for example binds to chemokines such as MIP-1α, MIP-1β and RANTES. These represent the six β-chemokine receptors that have currently been characterized.

The chemokine receptors share significant identity with each other. For example, CCR5 has significant identity to CCR2, sharing 71% identical amino acid residues. Its identity with other members of the family is about 50%. CCR3 shares a 62% amino acid identity with CCR1 and identity with the other characterized receptors that ranges between about forty and fifty percent. CCR3 and CCR5 do not show as much identity to each other as they do to other chemokine receptors. Similarly, they do not show a chemokine affinity pattern that is as similar to each other as it is to other members of the family. Yet, these two receptors facilitate entry of primary HIV-1 macrophage-tropic strains.

In the initial assays where CCR3 was not as highly expressed as CCR5, CCR5 displayed a broader apparent host range than CCR3. However, in more sensitive assays we have found that CCR3 facilitate HIV infection for all primary macrophage-tropic strains tested. Thus, CCR3 can interact with macrophage-tropic strains.

CCR5 is particularly involved with the following isolates: ADA, YU2, Br20-4, Br25-9, Rw20-5, Th966, TN243 and 89.6 More preferably the strains are ADA, YU2 BR20-4 and RW20-5.

CCR3 is particularly effective with the ADA and YU2 viruses and, to a lesser extent, with the 89.6 and ELI.

Whereas CCR5 is expressed in primary monocyte/macrophage, primary T cells and granulocyte precursors [Deng, H.K., et al., 1996; Alkhatib, G., et al., 1996] and CXCR4 is expressed in a broad range of

tissues and cell types including the brain and T cell [Feng, Y., et al, 1996], CCR3 expression appears more restricted, typically eosinophils.

Microglia are the major targets for HIV infection of the central nervous system. Microglia express CCR3 as well as CCR5 Price, R.W., 1996; Watkins, B.A., et al., 1996; Takahashi, K., et al., 1990). Astrocytes are also infected, but only at a very low level (Takahashi, K., et al., 1996; Harouse, J.M., et al., 1989; Tornatore C., et al., 1991). HIV-1 entry into microglia is CD4-dependent, (Jordan, et al., 1991) while entry into astrocytes and some neurally-derived cell lines is CD4-independent (Harouse, J.M., et al., 1989; Tornatore C., et al., 1991). HIV-1 viruses that infect the CNS are M-tropic HIV-1 isolates, which represent the majority of primary isolates (Watkins, B.A., et al., 1990; Korber, B.T.M., et al., 1994; Power, C., et al., 1994; Strizki, J.M., et al., 1996). Particular *env* sequences have been suggested as being associated with brain infection or dementia (Jirberm , et al., 1994; Power, C., et al., although specific determinants of HIV-1 neurotropism have not been identified.

We have discovered that macrophage tropic isolates use CCR5 and CCR3 as co-receptors to infect microglia efficiently, whereas T-tropic isolates use CXCR4. For example, blocking CCR5 or CCR3 can reduce microglia infection by M-tropic isolates by 70-80%.

It was previously reported that certain chemokines had different inhibitory effects on HIV activity. For example, RANTES was reported as having greater inhibitory activity than the other chemokines identified. This indicates that the known chemokines receptors will not typically be solely responsible for enhancing infection. This is because CCR5 has a greater sensitivity to MIP-1α than RANTES, yet RANTES exhibits a greater inhibitory activity than MIP-1α. Similarly CCR3 is responsive to RANTES but not to MIP-1α.

Further, the distribution of these receptors differs. For example CCR5 is primarily expressed in promyeloblastic cells, particularly KG-1A, CD4-positive, and CD8-positive human PBMC and cells of the myeloid lineage. CCR3 as discussed above is highly expressed in eosinophils with some expression in peripheral blood T lymphocytes. We have discovered that CCR3 is also expressed on dendritic cells, which is an important HIV reservoir. CCR3 is expressed at low levels on monocytes. The complete

characterization of the full tissue and cell-type distribution for these molecules awaits further studies.

Enhanced effectiveness in facilitating infections appears to be dependent upon the number of receptors expressed. Assays which measure receptor level can be used in monitoring HIV-infected and high risk individuals. Differences in the levels of expression of these receptors in different individuals can account for some of the differences observed in onset of AIDS in HIV-infected individuals. Thus, determining the level of these receptors in HIV-infected individuals can be an important tool in determining whether an individual is at a greater risk for enhanced risk of infection and onset of AIDS. This knowledge can be used in determining the type of treatment for that individual.

The determination of the number of receptors present on the cells of an individual can readily be accomplished by standard means, for example, using FACS analysis or analysis of RNA levels. The level can be compared to a reference level, which can be determined by standard means. For example, one can prepare averages for individuals exhibiting early onset of AIDS, standard onset of AIDS and delayed onset of AIDS. This can also be done with respect to risk of HIV infection. Moreover, one can also take into account the presence of chemokines such as RANTES, MIP-1α and/or MIP-1β in relationship to the level of CCR3 and/or CCR5 present. These assays are further discussed below.

Viral variation, particularly that found in the gp120 glycoprotein sequences (28,29), dictates the specific chemokine receptor that can be utilized as an entry cofactor. M-tropic HIV-1 variants that use the chemokine receptor CCR5 as a coreceptor predominate during the asymptomatic stages of infection [Alkhatib, G., et al., *Nature Med* 2:1244-1247; Deng, H.K., et al., *Nature* 381:661-666 (1996); Doranz, B., et al., *Cell* 85:1149-1158 (1996); Dragic, T., et al., *Nature* 381:667-673 (1996); Zhang, L., et al., *Nature* 383:768 (1996); Connor, R.I., et al., *J Exp Med* 185:621-628 (1997)]. CCR5 is expressed on T lymphocytes, monocytes/macrophages, brain microglia and dendritic cells Wu, L., et al., *J Exp Med*; Granelli-Piperno, A., et al., *J Exp Med* 184:2433-2438 (1996); Raport, C., et al., *J Biol Chem* 271:1761-1766 (1996); He, J., et al., *Nature* 385:645-649 (1997)]. Individuals with defects in CCR5 expression are relatively resistant to HIV-1

infection [Liu, R., et al., *Cell* 86:367-378 (1996); Dean, M., et al., *Science* 273:1856-1862 (1996); Samson, M., et al., *Nature* 382:722-725 (1996)], indicating the critical contribution of this chemokine receptor to virus transmission. Some M-tropic brain isolates of HIV-1 also use the chemokine receptor CCR3 as a coreceptor, consistent with the expression of CCR3 in brain microglia [He, J., et al., *Nature* 385:645-649 (1997)]. Later in the course of infection, T-tropic HIV-1 variants emerge that can use chemokine receptors, especially CXCR4, but also CCR3 and CCR2b, in addition to CCR5 [Zhang, L., et al., *Nature* 383:768 (1996); Connor, R.I., et al., *J Exp Med* 185:621-628 (1997); Simmons, G., et al., *J Virol* 70:8355-8360 (1996); Feng, Y., et al., *Science* 272:872-877 (1996); Schuitemaker, H. et al., *J Virol* 65:356-363 (1992)]. The emergence of these viruses has been suggested to coincide with a less favorable clinical prognosis [Schuitemaker, H. et al., *J Virol* 65:356-363 (1992)], perhaps through an expansion of the range of infectable CD-4 positive T cell subsets [Bleul, C., et al., *Proc Natl Acad Sci USA*].

Another preferred embodiment of this invention is in the diagnosis of susceptibility to HIV infection. The receptors, nucleotide sequences encoding receptors and antibodies that bind to receptors can be particularly useful for diagnosis of susceptibility to infection where higher levels of the receptors indicate an increased risk of infection. The nucleotide sequences are known, for example the sequence for CCR3 is available from GenBank/EMBL/DDB under Accession Nos. U 49727 and U51241.

Using any suitable technique known in the art, such as Northern blotting, quantitative PCR, etc. the nucleotide sequences of the receptors or fragments thereof can be used to measure levels of chemokine receptor RNA expression.

Alternatively, the antibodies of the invention can be used in standard techniques such as Western blotting to detect the presence of cells expressing receptors and using standard techniques, e.g. FACS or ELISA, to quantify the level of expression.

One can inhibit infection by blocking CCR3 and/or CCR5. This can be accomplished by a range of different approaches. For example, antibodies, decoys, small molecules, antagonists, etc. One preferred approach is the use of antibodies to these receptors. Antibodies to these

receptors can be prepared by standard means. For example, one can use single chain antibodies to target these receptors. An alternative strategy is to use CCR3 and CCR5 decoys. For example, one could prepare a decoy comprising the portion of these receptors present on the exterior of the cell membrane. Another strategy is to prepare soluble forms of these receptors using their known sequence. This can be done by standard means including using PCR to clone a gene, site- directed mutagenesis to make changes in the structure, deletions to make fragments, etc. as discussed below.

We have discovered that the HIV-1 gp120 envelope glycoprotein interacts with the CD4 receptor resulting in a conformational change in the molecule which enhances the binding affinity of the gp120 protein for the chemokine receptor. This is exemplified herein with CCR5. Our results demonstrate that gp120 glycoproteins will specifically interact with chemokine receptors, and that the interaction is dramatically enhanced by the formation of a complex with CD4. Interaction with the chemokine receptors is necessary for efficient membrane fusion and thereby viral infection. Accordingly, a new group of compounds that can be used to inhibit infection (membrane fusion) is disclosed. For example, gp120 derivatives containing the chemokine binding site attached to a soluble CD4 molecule will have enhanced binding affinity to the chemokine receptors over the uncomplexed gp120. Molecules that preferentially bind to these binding sites on gp120 will also prevent membrane fusion, for example, we have shown that the broadly neutralizing monoclonal antibody 17b can inhibit binding of gp120, e.g. the binding of the glycoprotein to CCR5.

Compounds that affect this interaction can be directly screened for example using a direct binding assay such as exemplified in Figure 12. For example, one can label, e.g. radioactive or fluorescent, a gp120 protein or derivative and add soluble CD4. There are various soluble CD4s known in the art including a two-domain (D1D2 sCD4) and a four-domain version. The labeled gp120, or derivative e.g. a conformational deletion such as YU2ΔC1ΔV1/2ΔC5 protein and soluble CD4 can be added to a medium containing a cell line expressing a chemokine receptor that that derivative will bind to. In this example, the derivative will bind to CCR5. Alternatively, when using a derivative from a T cell tropic gp120 one would use a cell line

that expresses CXCR4. Binding of the protein can then be directly measured. The compound of interest can be added before or after the addition of the labeled gp120 or derivative and the effect of the derivative on binding can be determined by comparing the degree of binding in that situation against a base line standard with that gp120 or derivative, not in the presence of the compound.

One can use a stabilized complex of soluble CD4 and a gp120 molecule or a conformational derivative thereof as a decoy.

A preferred assay uses the labeled gp120, or derivative, for example a gp120 protein derived from an M-tropic strain such as JR-FL, iodinated using for instance solid phase lactoperoxidase (in one example having a specific activity of 20 μCi/μg). The cell line containing the chemokine receptor in this example would be a CCR5 cell line, e.g. L1.2 or membranes thereof. Soluble CD4 would be present. For screening small molecule antagonists, cell membranes are preferable, although similar procedures work with whole cells.

To test for compounds that affect binding the test molecule is added to the solution. Unlabeled gp120 can serve as a control. For example in a binding reaction in a final volume of 100 μl, 25 μl of soluble CD4 diluted in binding buffer (e.g. 50mM HEPES, pH 7.2, 1 mM $CaCl_2$, 5 mM $MgCl_2$ and 0.5% BSA) are added. 25 μl of binding buffer (for total binding) unlabeled gp120 at a final concentration of 100 nM (for non-specific binding), or test compounds at the desired concentrations are added. 25 μl of membranes (or whole cells), detected in binding buffer at the desired concentration are added, followed by 25 μl of labeled (e.g. ^{125}I-labeled) gp120 at a final concentration of 0.1 nM. The contents are mixed and incubated at room temperature for 45-60 minutes. The reactions are then stopped. For example by transferring the mixture to GFB filter plates, pretreated with 0.3% PEI, washed (e.g., 2-3 times) with binding buffer containing e.g. 0.5 M NaCl in an automated cell harvester. The plates are dried, for example by heat lamp and the activity measured -- e.g. a MicroScint scintillation fluid added and the radioactivity counted for example on a β-counter. See Figures 13-15.

The binding assay can be adapted depending upon precisely what is being tested.

As used herein, the conformational derivative must contain a sufficient number of amino acid residues to define the binding site of the gp120 to the chemokine receptor and a sufficient number of amino acids to maintain the conformation of the peptide in a conformation that approximates that of wild-type gp120 bound to soluble CD4 with respect to the chemokine receptor binding site.

In one preferred embodiment, the derivative also contains a CD4 binding site (e.g. from the C3 region residues 368 and 370, and from the C4 region residues 427 and 457). As discussed herein, we have now discovered that the chemokine binding site is a discontinuous binding site that includes portions of the C2, C3, C4 and V3 regions. We also show herein certain examples of the gp120 derivatives that contain the binding site. By deletion of non-essential portions of the gp120 derivatives -- such as deletions of portions of non-essential variable regions (e.g. V1/V2) or protein in these constant regions (e.g. C1, C5) one can increase exposure of the chemokine binding site thereby enhancing the ability of the gp120 derivative to bind to the chemokine receptor, thereby inhibiting viral entry. Removal of these regions is done while requiring the derivative to retain an overall conformation approximating that of the wild-type protein with respect to the native gp120 chemokine binding region when complexed to CD4. In addition, one can remove glycosylation sites that are disposable for proper folding. Maintaining conformation can be accomplished by using linker residues that permit potential turns in the structure of the gp120 derivative to maintain the overall three-dimensional structure. Preferred amino acid residues that can be used as linker include Gly and Pro. Other amino acids can also be used as part of the linker, e.g. Ala. Examples on how to prepare such peptides are described more fully in Wyatt, R., *et al. J. of Virol.* 69:5723-5733 (1995); Thali, M., *et al., J. of Virol.* 67:3978-3988 (1993); and U.S. Application Serial No. 07/858,165 filed March 26, 1992 which are incorporated herein by reference. See for example Wyatt which teaches how to prepare V1/V2 deletions that retain the stem portion of the loop.

In one embodiment the gp120 derivative is designed to be permanently attached at the CD4 binding site to sufficient domains of CD4 to create a conformation of the chemokine binding site approximating that of the native gp120 CD4 complex.

An alternative gp120 derivative is one wherein the linkers used result in a conformation for the derivative so that the discontinuous binding site with the chemokine receptor approximates the conformation of the discontinuous binding site for the chemokine receptor in the wild-type gp120/CD4 complex. These derivatives can readily be made by the person of ordinary skill in the art based upon the above described methodologies and screened in the assays shown herein to ensure that proper binding is obtained.

Stabilized forms of these complexes can readily be made, for example, by conjugates such as a poly(alkylene oxide) conjugate. The conjugate is preferably formed by covalently bonding the hydroxyl terminals of the poly(alkylene oxide) and a free amino group in the gp120 derivative that will not affect the conformation of the discontinuous binding site. Other art recognized methods of conjugating these materials include amide or ester linkages. Covalent linkage as well as non-covalent conjugation such as lipophilic or hydrophilic interactions can be used.

The conjugate can be comprised of non-antigenic polymeric substances such as dextran, polyvinyl pyrrolidones, polysaccharides, starches, polyvinyl alcohols, polyacryl amides or other similar substantially non-immunogenic polymers. Polyethylene glycol(PEG) is preferred. Other poly(alkylenes oxides) include monomethoxy-polyethylene glycol polypropylene glycol, block copolymers of polyethylene glycol, and polypropylene glycol and the like. The polymers can also be distally capped with C1-4 alkyls instead of monomethoxy groups. The poly(alkylene oxides) used must be soluble in liquid at room temperature. Thus, they preferably have a molecular weight from about 200 to about 20,000 daltons, more preferably about 2,000 to about 10,000 and still more preferably about 5,000.

One can administer these stabilized compounds to individuals by a variety of means. For example, these compounds can be included in vaginal foams or gels that are used as preventives to avoid infection and applied before people have sexual contact.

The peptides when used for administration are prepared under aseptic conditions with a pharmaceutically acceptable carrier or diluent.

Doses of the pharmaceutical compositions will vary depending upon the subject and upon the particular route of administration used. Dosages can range from 0.1 to 100,000µg/kg a day, more preferably 1 to 10,000µg/kg.

Routes of administration include oral, parenteral, rectal, intravaginal, topical, nasal, ophthalmic, direct injection, etc.

Changes in the viral envelope glycoproteins, in particular in the third variable (V3) region of the gp120 exterior envelope glycoprotein, determine tropism-related phenotypes (Cheng-Mayer *et al.*, 1990; O'Brien *et al.*, 1990; Hwang *et al.*, Westervelt *et al.*, 1992; Chesebro *et al.*, 1992; Willey *et al.*, 1994). Amino acid changes in the V3 region (Helseth *et al.*, 1990; Freed *et al.*, 1991; Ivanoff *et al.*, 1991; Bergeron *et al.*, 1992; Grimaila *et al.*, 1992; Page *et al.*, 1992; Travis *et al.*, 1992) and the binding of antibodies to this domain (Putney *et al.*, 1986; Goudsmit *et al.*, 1988; Linsley *et al.*, 1988; Rusche *et al.*, 1988; Skinner *et al.*, Javeherian *et al.*, 1989) have been shown to disrupt a virus entry process other than CD4 binding. Accordingly, one can create derivatives and change the phenotype for a particular receptor by substituting V3 loops.

One can inhibit infection by directly blocking CCR3 and/or CCR5. This can be accomplished by a range of different approaches. For example, antibodies, decoys, small molecules, antagonists, etc. One preferred approach is the use of antibodies to the binding site for these chemokine receptors. Antibodies to these receptors can be prepared by standard means using the gp120 derivatives and gp120/CD4 complexes. For example, one can use single chain antibodies to target these binding sites. An alternative strategy is to use the stabilized gp120/CD4 complexes as decoys.

As used herein the inhibition of HIV infection means that as compared to a control situation infection is reduced, inhibited or prevented. Infection is preferably at least 20% less, more preferably at least 40% less, even more preferably at least 50% less, still more preferably at least 75% less, even more preferably at least 80% less, and yet more preferably at least 90% less than the control.

The isolated nucleotide sequences and isolated polypeptides of the invention encoding receptors can be mutagenized by any of several standard

methods including treatment with hydroxylamine, passage through mutagenic bacterial strains, etc. The mutagenized sequences can then be classified "wild type" or "non-wild type" depending whether it will still facilitate infectivity or not.

Mutagenized sequences can contain point mutations, deletions, substitutions, rearrangements etc. Mutagenized sequences can be used to define the cellular function of different regions of the receptors they encode, and to define the portions of the receptor that facilitate HIV-1 infection. This information can be used to assist in the design of small molecules or peptides mimicking the HIV-interactive part of the chemokine receptor. These small molecule/peptides can be used to inhibit HIV-1 infection. As used herein the inhibition of HIV-infection means that as compared to a control situation infection is reduced, inhibited or prevented. Infection is at least 20% less, preferably at least 40% less, more preferably at least 50% less, still more preferably at least 75% less, even more preferably at least 80% less, and yet more preferably at least 90% less than the control.

Another approach is to use small molecules that will selectively bind to one of the receptors. Some preferred small molecules include the chemokines themselves (e.g. eotaxin, RANTES, MCP-1, MIP-1α and/or MIP-1β),fragments of chemokines, preferably surface fragments, and smaller molecules or peptides the mimic the chemokines. Such molecules and peptides can be synthesized by known techniques.

We have also discovered certain coreceptors involved in the binding of another primate immunodeficiency virus--SIV. While SIV have been shown to use CCR5 as a coreceptor, other receptors for the virus have not previously been reported. We have found that two orphan seven-transmembrane segment receptors, gpr1 and gpr15, serve as coreceptors for SIV, and are expressed in alveolar macrophages. gpr15 which is also expressed in $CD4^+$ T lymphocytes is the more efficient.

The SIV coreceptors, gpr1 and gpr15, are expressed in U87 and CEMx174 cells, respectively. CEMx174 supports SIV entry but lacks CCR5 and does not support efficient entry of HIV-1 viruses using CCR5. The neuroglioma cell line U87, stably transfected with CD4 similarly supports entry of SIV_{mac} 239 but does not allow for efficient entry of known HIV-1 viruses. We further found that the HIV-1 strains ADA and YU2 weakly use

gpr15. This may be an inadvertent consequence of similarities in the amino-terminal regions of gpr15 and CCR5, or it may indicate an adaptation to these or a related receptor that occurs in some HIV-1 subsets.

In primary structure, gpr1 and gpr15 resemble the angiotensin II receptor and the orphan receptors dez and apj more than they do any of the known chemokine receptors [Marchese, A., et al., *Genomics* 23:609-618 (1994); Heiber, M., et al., *Genomics* 32:462-465 (1996)]. Gpr15, like dez and gpr1, lacks the cysteines in the amino-terminal region and the third extracellular loop that, in the chemokine receptors, are thought to be disulfide linked. It is interesting that, despite the general sequence divergence of gpr15/gpr1 and other identified primate immunodeficiency virus coreceptors, the gpr15 and gpr1 amino termini contain three tyrosines that align with similarly-positioned tyrosines in CCR5 (See Figure 15). Alteration of these tyrosines has been shown to decrease the efficiency with which CCR5 supports the entry of SIV and macrophage-tropic HIV-1 isolates (M. Farzan, H. Choe and J. Sodroski, unpublished observations). The identification of gpr 15 and gpr1 as SIV coreceptors suggests a greater range and complexity of coreceptors for the primate immunodeficiency viruses than heretofore described. Comparative studies of these divergent coreceptors with the known coreceptors for these viruses should assist the identification of common structural elements in 7-TMS proteins that serve as viral entry cofactors.

A molecule that binds to at least one of the tyrosine residues present in the amino terminus of the coreceptors is a preferred molecule for interfering with HIV entry. One class of molecules are antibodies, for example a single chain antibody. One can use a segment of the receptor containing at least one of the tyrosine residues to generate the antibody. For example, one can use fragments of SEQ ID NOS: 1-4 that are at least 6 amino acid residues in length and contain at least one of the conserved tyrosine residues, preferably at least two of the tyrosines residues and more preferably all three conserved tyrosine residues. For example, one could use SEQ ID NO:4 or fragments thereof to generate an antibody by standard means. Thereafter using the binding assay described herein one can select those antibodies generated that most effectively inhibit chemokine binding such as CCR5 binding. Another class of molecules is a small molecule.

One preferred use of these compounds is to minimize the risk of HIV transmission. These compounds can be included in ointments, foams, creams that can be used during sex. For example, they can be administered preferably prior to or just after sexual contact such as intercourse. One preferred composition would be a vaginal foam containing one of the compounds. Preferably the compound would be a decoy or blocker, for example, a small molecule that binds to the CCR3 receptor. Another use would be in systemic administration to block HIV-1 replication in the blood and tissues. The compound could also be administered in combination with other HIV treatments.

Another strategy is to express antibodies to these receptors in infected individuals intracellularly. This can be done by the method of Marasco and Haseltine set forth in WO94-02610 (PCT/US93/06735 filed July 16, 1993) published February 3, 1994.

In addition, additional compounds that bind to these receptors and thus interfere with their ability to facilitate HIV infection can readily be screened for. For example, one can select cells expressing high numbers of these receptors, plate them; e.g. add labeled gp120 and CD4 and screen for compounds or combinations of compounds that will interact with, e.g. binding of, these receptors by standard techniques. Alternatively, one can use known techniques to prepare cells that will express these receptors and use those cells in drug screens. In particular, the ability of drugs to block HIV-1 infection or syncytium formation can be screened using assays similar to those showing in Figures 1-5.

One can prepare a drug screen using a cell line or cell membrane expressing CD4 and either CCR3 or CCR5. Preferably, the surface receptors would only be CD4 and either CCR3 and/or CCR5. In another embodiment one would use a cell line or cell membrane that expresses CD4 and the only other chemokine co-receptor would be CCR3 and/or CCR5. Thus, one can determine if the compounds tested affect infectivity by HIV-1. Such a method can be used to select molecules that specifically affect the pathway. These molecules may be combined with other drugs, for example, for their combined or synergistic effects. In contrast, when comparing CD4 cells there can be a variety of other factors affecting such cells, thus, such a comparison does not provide the same data.

One can also prepare cell lines stably expressing CCR3 or CCR5, by themselves, or with CD4. Such cells can be used for a variety of purposes including an excellent source of antigen for preparing a range of antibodies using techniques well known in the art.

By creating cells expressing these receptors, one can enhance the range of cells these primary HIV-1 macrophage tropic strains can infect. For example, one can use CD4 expressing cell lines or vector systems cotransfecting the genes encoding CD4 and at least one of these receptors.

One of the problems that has been encountered in in vivo testing compounds that affect HIV-1 is the relatively small number of animals that can be infected by HIV. While systems such as a chimeric virus comprising SIV and HIV (SHIV) have extended the number of animal models that can be used, this approach is primarily directed to systems that use other primates. Now one can prepare transgenic animals that have cells that express CD4 and at least CCR5 or CCR3 to further extend the range of animals susceptible to HIV-1 infection. This permits one to create a much broader range of animal models.

A "transgenic animal" is an animal having cells that contain DNA which has been artificially inserted into a cell, which DNA becomes part of the genome of the somatic cells and/or the germ line of the animal that develops from that cell. The preferred DNA contains nucleotide sequences that are homologous to human CD4, CCR3 and/or CCR5 genes. These sequences may be entirely foreign to the transgenic animal or may even be identical to the homologous gene of the animal, but which is inserted into the animal's genome at a location which differs from that of the natural copy. Transgenic animals can provide good model systems for studying the development of AIDS, the effects of potential therapeutic reagents, and the safety (e.g. toxicity, carcinogenicity) of such agents administered to the animals.

Therapeutic and Pharmaceutic Compositions

An exemplary pharmaceutical composition is a therapeutically effective amount of a decoy, antibody etc. that affects the ability of the receptor to facilitate HIV infection optionally included in a pharmaceutically-acceptable and compatible carrier. The term "pharmaceutically-acceptable and compatible carrier" as used herein, and described more fully below,

includes (i) one or more compatible solid or liquid filler diluents or encapsulating substances that are suitable for administration to a human or other animal, and/or (ii) a system, such as a retroviral vector, capable of delivering the molecule to a target cell. In the present invention, the term "carrier" thus denotes an organic or inorganic ingredient, natural or synthetic, with which the molecules of the invention are combined to facilitate application. The term "therapeutically-effective amount" is that amount of the present pharmaceutical compositions which produces a desired result or exerts a desired influence on the particular condition being treated. Various concentrations may be used in preparing compositions incorporating the same ingredient to provide for variations in the age of the patient to be treated, the severity of the condition, the duration of the treatment and the mode of administration.

The term "compatible", as used herein, means that the components of the pharmaceutical compositions are capable of being commingled with a small molecule, nucleic acid and/or polypeptides of the present invention, and with each other, in a manner such that does not substantially impair the desired pharmaceutical efficacy.

Dose of the pharmaceutical compositions of the invention will vary depending on the subject and upon particular route of administration used. Dosages can range from 0.1 to 100,000 μg/kg per day, more preferably 1 to 10,000 μg/kg. By way of an example only, an overall dose range of from about, for example, 1 microgram to about 300 micrograms might be used for human use. This dose can be delivered at periodic intervals based upon the composition. For example on at least two separate occasions, preferably spaced apart by about 4 weeks. Other compounds might be administered daily. Pharmaceutical compositions of the present invention can also be administered to a subject according to a variety of other, well-characterized protocols. For example, certain currently accepted immunization regimens can include the following: (i) administration times are a first dose at elected date; a second dose at 1 month after first dose; and a third dose at 5 months after second dose. See Product Information, *Physician's Desk Reference*, Merck Sharp & Dohme (1990), at 1442-43. (e.g., Hepatitis B Vaccine-type protocol); (ii) Recommended administration for children is first dose at elected date (at age 6 weeks old or older); a second dose at 4-8 weeks after

first dose; a third dose at 4-8 weeks after second dose; a fourth dose at 6-12 months after third dose; a fifth dose at age 4-6 years old; and additional boosters every 10 years after last dose. See Product Information, *Physician's Desk Reference*, Merck Sharp & Dohme (1990), at 879 (e.g., Diptheria, Tetanus and Pertussis-type vaccine protocols). Desired time intervals for delivery of multiple doses of a particular composition can be determined by one of ordinary skill in the art employing no more than routine experimentation.

The small molecules and polypeptides of the invention may also be administered *per se* (neat) or in the form of a pharmaceutically acceptable salt. When used in medicine, the salts should be pharmaceutically acceptable, but non-pharmaceutically acceptable salts may conveniently be used to prepare pharmaceutically acceptable salts thereof and are not excluded from the scope of this invention. Such pharmaceutically acceptable salts include, but are not limited to, those prepared from the following acids: hydrochloric, hydrobromic, sulfuric, nitric, phosphoric, maleic, acetic, salicylic, p-toluene-sulfonic, tartaric, citric, methanesulphonic, formic, malonic, succinic, naphthalene-2-sulfonic, and benzenesulphonic. Also, pharmaceutically acceptable salts can be prepared as alkaline metal or alkaline earth salts, such as sodium, potassium or calcium salts of the carboxylic acid group. Thus, the present invention also provides pharmaceutical compositions, for medical use, which comprise nucleic acid and/or polypeptides of the invention together with one or more pharmaceutically acceptable carriers thereof and optionally any other therapeutic ingredients.

The compositions include those suitable for oral, rectal, intravaginal, topical, nasal, ophthalmic or parenteral administration, all of which may be used as routes of administration using the materials of the present invention. Other suitable routes of administration include intrathecal administration directly into spinal fluid (CSF), direct injection onto an arterial surface and intraparenchymal injection directly into targeted areas of an organ. Compositions suitable for parenteral administration are preferred. The term "parenteral" includes subcutaneous injections, intravenous, intramuscular, intrasternal injection or infusion techniques.

The compositions may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. Methods typically include the step of bringing the active ingredients of the invention into association with a carrier which constitutes one or more accessory ingredients.

Compositions of the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets, tablets or lozenges, each containing a predetermined amount of the nucleic acid and/or polypeptide of the invention in liposomes or as a suspension in an aqueous liquor or non-aqueous liquid such as a syrup, an elixir, or an emulsion.

Preferred compositions suitable for parenteral administration conveniently comprise a sterile aqueous preparation of the molecule of the invention which is preferably isotonic with the blood of the recipient. This aqueous preparation may be formulated according to known methods using those suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation may also be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for example as a solution in 1,3-butane diol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil may be employed including synthetic mono- or diglycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectibles.

Antibodies

The term "antibodies" is meant to include monoclonal antibodies, polyclonal antibodies and antibodies prepared by recombinant nucleic acid techniques that are selectively reactive with polypeptides encoded by eukaryotic nucleotide sequences of the present invention. The term "selectively reactive" refers to those antibodies that react with one or more antigenic determinants of CCR3 or CCR5, or gp120 and/or CD4 and do not react with other polypeptides. Antigenic determinants usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and have specific three dimensional structural

characteristics as well as specific charge characteristics. Antibodies can be used for diagnostic applications or for research purposes.

For example, antibodies may be raised against amino-terminal (N-terminal) or carboxyl-terminal (C-terminal) peptides of a polypeptide encoded by CCR3, CCR5. Most preferably one selects an exposed cell-surface epitope of one of these receptors.

One approach is to isolate a peptide sequence that contains an antigenic determinant for use as an immunogen. This peptide immunogen can be attached to a carrier to enhance the immunogenic response. Although the peptide immunogen can correspond to any portion of a polypeptide encoded by a eukaryotic nucleotide sequence of the invention, certain amino acid sequences are more likely than others to provoke an immediate response, for example, an amino acid sequence including the N- or C-terminus of a polypeptide encoded by a gene that contains nucleotide sequences of the invention. Preferably one can use a cell line expressing only CCR3, select those cells with the highest levels of expression and use the whole cell as an antigen.

For example, cDNA clone encoding a CCR3, CCR5 or a fragment thereof may be expressed in a host using standard techniques (see above; see Sambrook et al., Molecular Cloning; A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, New York: 1989) such that 5-20% of the total protein that can be recovered from the host is the desired protein. Recovered proteins can be electrophoresed using PAGE and the appropriate protein band can be cut out of the gel. The desired protein sample can then be eluted from the gel slice and prepared for immunization. Alternatively, a protein of interest can be purified by using conventional methods such as, for example, ion exchange hydrophobic, size exclusion, or affinity chromatography.

Once the protein immunogen is prepared, mice can be immunized twice intraperitoneally with approximately 50 micrograms of protein immunogen per mouse. Sera from such immunized mice can be tested for antibody activity by immunohistology or immunocytology on any host system expressing such polypeptide and by ELISA with the expressed polypeptide. For immunohistology, active antibodies of the present invention can be identified using a biotin-conjugated anti-mouse

immunoglobulin followed by avidin-peroxidase and a chromogenic peroxidase substrate. Preparations of such reagents are commercially available; for example, from Zymad Corp., San Francisco, California. Mice whose sera contain detectable active antibodies according to the invention can be sacrificed three days later and their spleens removed for fusion and hybridoma production. Positive supernatants of such hybridomas can be identified using the assays described above and by, for example, Western blot analysis.

To further improve the likelihood of producing an antibody as provided by the invention, the amino acid sequence of polypeptides encoded by a eukaryotic nucleotide sequence of the present invention may be analyzed in order to identify portions of amino acid sequence which may be associated with increased immunogenicity. For example, polypeptide sequences may be subjected to computer analysis to identify potentially immunogenic surface epitopes. Such computer analysis can include generating plots of antigenic index, hydrophilicity, structural features such as amphophilic helices or amphophilic sheets and the like.

For preparation of monoclonal antibodies directed toward polypeptides encoded by a eukaryotic nucleotide sequence of the invention, any technique that provides for the production of antibody molecules by continuous cell lines may be used. For example, the hybridoma technique originally developed by Kohler and Milstein (Nature, 256: 495-497, 1973), as well as the trioma technique, the human B-cell hybridoma technique (Kozbor et al., Immunology Today, 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies, and the like, are within the scope of the present invention. See, generally Larrick et al., U.S. Patent 5,001,065 and references cited therein. Further, single-chain antibody (SCA) methods are also available to produce antibodies against polypeptides encoded by a eukaryotic nucleotide sequence of the invention (Ladner et al. U.S. patents 4,704,694 and 4,976,778).

The monoclonal antibodies may be human monoclonal antibodies or chimeric human-mouse (or other species) monoclonal antibodies. The present invention provides for antibody molecules as well as fragments of such antibody molecules.

Those of ordinary skill in the art will recognize that a large variety of possible moieties can be coupled to the resultant antibodies or to other molecules of the invention. See, for example, "Conjugate Vaccines", Contributions to Microbiology and Immunology, J.M. Cruse and R.E. Lewis, Jr (eds), Carger Press, New York, (1989), the entire contents of which are incorporated herein by reference.

Coupling may be accomplished by any chemical reaction that will bind the two molecules so long as the antibody and the other moiety retain their respective activities. This linkage can include many chemical mechanisms, for instance covalent binding, affinity binding, intercalation, coordinate binding and complexation. The preferred binding is, however, covalent binding. Covalent binding can be achieved either by direct condensation of existing side chains or by the incorporation of external bridging molecules. Many bivalent or polyvalent linking agents are useful in coupling protein molecules, such as the antibodies of the present invention, to other molecules. For example, representative coupling agents can include organic compounds such as thioesters, carbodiimides, succinimide esters, diisocyanates, glutaraldehydes, diazobenzenes and hexamethylene diamines. This listing is not intended to be exhaustive of the various classes of coupling agents known in the art but, rather, is exemplary of the more common coupling agents. (See Killen and Lindstrom 1984, "Specific killing of lymphocytes that cause experimental Autoimmune Myasthenia Gravis by toxin-acetylcholine receptor conjugates." Jour. Immun. 133:1335-2549; Jansen, F.K., H.E. Blythman, D. Carriere, P. Casella, O. Gros, P. Gros, J.C. Laurent, F. Paolucci, B. Pau, P. Poncelet, G. Richer, H. Vidal, and G.A. Voisin. 1982. "Immunotoxins: Hybrid molecules combining high specificity and potent cytotoxicity". Immunological Reviews 62:185-216; and Vitetta et al., supra).

Preferred linkers are described in the literature. See, for example, Ramakrishnan, S. et al., Cancer Res. 44:201-208 (1984) describing use of MBS (M-maleimidobenzoyl-N-hydroxysuccinimide ester). See also, Umemoto et al. U.S. Patent 5,030,719, describing use of halogenated acetyl hydrazide derivative coupled to an antibody by way of an oligopeptide linker. Particularly preferred linkers include: (i) EDC (1-ethyl-3-(3-dimethylamino-propyl) carbodiimide hydrochloride; (ii) SMPT (4-succinimidyloxycarbonyl-

alpha-methyl-alpha-(2-pyridyl-dithio)-toluene (Pierce Chem. Co., Cat. (21558G); (iii) SPDP (succinimidyl-6 [3-(2-pyridyldithio) propionamido] hexanoate (Pierce Chem. Co., Cat #21651G); (iv) Sulfo-LC-SPDP (sulfosuccinimidyl 6 [3-(2-pyridyldithio)-propianamide] hexanoate (Pierce Chem. Co. Cat. #2165-G); and (v) sulfo-NHS (N-hydroxysulfo-succinimide: Pierce Chem. Co., Cat. #24510) conjugated to EDC.

The linkers described above contain components that have different attributes, thus leading to conjugates with differing physio-chemical properties. For example, sulfo-NHS esters of alkyl carboxylates are more stable than sulfo-NHS esters of aromatic carboxylates. NHS-ester containing linkers are less soluble than sulfo-NHS esters. Further, the linker SMPT contains a sterically hindered disulfide bond, and can form conjugates with increased stability. Disulfide linkages, are in general, less stable than other linkages because the disulfide linkage is cleaved in vitro, resulting in less conjugate available. Sulfo-NHS, in particular, can enhance the stability of carbodimide couplings. Carbodimide couplings (such as EDC) when used in conjunction with sulfo-NHS, forms esters that are more resistant to hydrolysis than the carbodimide coupling reaction alone.

Antibodies of the present invention can be detected by appropriate assays, e.g., conventional types of immunoassays. For example, a sandwich assay can be performed in which the receptor or fragment thereof is affixed to a solid phase. Incubation is maintained for a sufficient period of time to allow the antibody in the sample to bind to the immobilized polypeptide on the solid phase. After this first incubation, the solid phase is separated from the sample. The solid phase is washed to remove unbound materials and interfering substances such as non-specific proteins which may also be present in the sample. The solid phase containing the antibody of interest bound to the immobilized polypeptide of the present invention is subsequently incubated with labeled antibody or antibody bound to a coupling agent such as biotin or avidin. Labels for antibodies are well-known in the art and include radionuclides, enzymes (e.g. maleate dehydrogenase, horseradish peroxidase, glucose oxidase, catalase), fluors (fluorescein isothiocyanate, rhodamine, phycocyanin, fluorescamine), biotin, and the like. The labeled antibodies are incubated with the solid and the label bound to the solid phase is measured, the amount of the label detected

serving as a measure of the amount of anti-urea transporter antibody present in the sample. These and other immunoassays can be easily performed by those of ordinary skill in the art.

The following Examples serve to illustrate the present invention, and are not intended to limit the invention in any manner.

EXAMPLES

Primary macrophage-tropic and laboratory-adapted human immunodeficiency viruses type 1 (HIV-1) require particular chemokine receptors, CCR5 and CXCR4, respectively, in addition to the primary receptor, CD4, for efficient entry into target cells [Feng, Y., et al., Science 272:872-877 (1996); Choe, H., et al., Cell 85:1135-1148 (1996); Doranze, B.J., et al., *Cell* 85:1149-1158 (1996); Dragic, T., et al., *Nature* 381:661-666 (1996); Deng, H., et al., *Nature* 381:661-666 (1996)]. The following data demonstrates that a complex of the exterior envelope glycoprotein, gp120, of macrophage-tropic primary HIV-1 and soluble CD4 interacts specifically with CCR5 and inhibits the binding of the natural CCR5 ligands, MIP-1α and MIP-1β [Samson, M., et al., *Biochemistry* 271:3362-3367 (1996); Raport, C., et al., *J Biol Chem* 271:17161-17166 (1996)]. The apparent affinity of the gp120-CCR5 interaction was dramatically lower in the absence of soluble CD4. Additionally, in the absence of gp120, an interaction between a two-domain CD4 fragment and CCR5 was observed. A gp120 fragment retaining the CD4 binding site and overlapping epitopes was able to interact with CCR5 only if the V3 loop, which can specify HIV-1 tropism and chemokine receptor choice [Choe, H., et al., Cell 85:1135-1148 (1996); Cheng-Mayer, C., et al., *J Virol* 64:4390-4398 (1990); Chesebro, B., et al., *J Virol* 65:5782-5789 (1991); Hwang, S., et al., *Science* 253:71-74 (1991); Westervelt, P., et al., *J Virol* 66:2577-2582 (1992)], was also present on the molecule. Neutralizing antibodies directed against either CD4-induced or V3 epitopes on gp120 blocked the interaction of gp120 CD4 complexes with CCR5. These results indicate that HIV-1 attachment to CD4 creates a high-affinity binding site for a chemokine receptor such as CCR5, leading to membrane fusion and virus entry.

Cells

The murine pre-B lymphoma line, L1.2, was stably transfected with CCR5 cDNA, tagged at the N-terminus with a FLAG epitope (Kodak), in the

pMRB101 expression vector, as described [Ponath, P., et al., *J Exp Med* 183:2437-2448 (1996)]. The pMRB101 plasmid is a derivative of Ee6hcmvbglii that contains the E. coli gpt gene and was kindly provided by Martin Robinson (CellTech). The cell surface expression of CCR5 was monitored by staining with an anti-FLAG antibody, and cells with a high level of CCR5 expression were selected by several rounds of limiting dilution and rescreening. L1.2 cells stably expressing CCR1 were kindly provided by James Campbell and Eugene Butcher (Stanford University). Scatchard analysis of MIP-1α binding to CCR1-L1.2 cells revealed a dissociation constant of 8 nM and 2 x 10^4 binding sites per cell (S. Qin, unpublished results).

Chemokine Binding and Competition Assays

^{125}I-labeled human MIP-1α and MIP-1β and unlabeled chemokine were purchased from DuPont NEN (Boston, MA) and Peprotech (Rocky Hill, NJ), respectively. CCR5F-L1.2 cells were washed and resuspended in binding buffer (50 mM HEPES, pH7.5, 1 mM $CaCl_2$, 5mM $MgCl_2$ and 0.5% BSA) at a concentration of 5 x 10^8 cells/ml. For binding and competition studies, which were conducted in a final volume of 100 µl, gp120 glycoproteins were mixed with soluble CD4 on ice for 5-10 minutes, after which monoclonal antibodies were added if appropriate. After another 5-10 minutes, 25 µl of cell suspension (1.25 x 10^5 cells) was added, followed by radiolabeled chemokine (final concentration 0.1 nM). The reactions were then incubated at 37° C for 30-45 minutes and stopped by transferring the mixture to GFB filter plates, which were then washed twice with binding buffer containing 0.5 M NaCl. The plates were dried, immersed in MicroScint scintillation fluid, and counted. The total binding was determined by including only radiolabeled chemokine and cells in the reaction. Non-specific (background) binding was determined in the presence of 100 nM unlabeled chemokine. The percentage of inhibition was calculated using the following formula:

$$\% \text{ Inhibition Ligand Binding} = 100 - 100 \times \frac{(S-B)}{(T-B)},$$

where S is the test sample, B is the background, and T is the total binding. Each experiment was performed twice with duplicates.

Recombinant Glycoproteins

All of the gp120glycoproteins were produced from stably transfected Drosophila Schneider 2 cells, using the pMt vector and selectable marker, pc hygro [Culp, J.S., et al., *Biotechnology* 9:173-177 (1991)]. The JR-FL and BAL gp120 proteins were previously described [Ivey-Hoyle, M., *Proc Natl Acad Sci USA* 88:512-516 (1991)] and were generously provided by Dr. Raymond Sweet (SmithKline Beecham). The BAL gp120 derivative contains an amino terminal deletion of 32 residues compared with the wild-type gp120 glycoprotein. The YU2 glycoproteins were produced from a chimeric *env* gene, containing YU2 sequences from Bgl II (nucleotide 6620)to BGl II (nucleotide 7200) in an HXBc2 background. The ΔC1 proteins contain deletions up to and including residue 82. The ΔV1/2 and ΔV3 proteins contain deletions equivalent to the Δ128-194 and Δ298-329 deletions, respectively, previously described for the HXBc2 glycoprotein [Wyatt, R., et al., *J Virol* 69:5723-5733 (1995)]. The ΔC5 proteins contain a deletion of the carboxy-terminal 19 residues of the mature gp120 glycoprotein. All gp120 proteins utilize the tissue plasminogen activator signal sequence for translocation into the endoplasmic reticulum. Protein expression of recombinant YU2 and HXBc2 derivatives was induced by transfer of Drosophila lines into serum-free medium containing 750 mM $CuSO_4$ for seven days at 25° C. Recombinant proteins were purified by passage of cell supernatants over an F105 monoclonal antibody column, which was extensively washed with PBS containing 500 mM NaCl and then reequilibrated in PBS containing 150 mM NaCl. The gp120 glycoproteins were eluted with 100 mM glycine-HCL, pH 2.8 and fractions were immediately neutralized with 1 M Tris base. The gp120 glycoproteins were concentrated using Centriprep 30 spin filters (Amicon), and resuspended in PBS containing protease inhibitors. Protein concentrations were determined by comparison with commercially available gp120 (Agmed) on Coomassie blue-stained SDS-PAGE gels. All of the gp120 preparations were homogenous, with the exception of the BAL gp120 preparation, in which approximately 5 percent of the protein was proteolytically cleaved.

Soluble CD4 proteins [Arthos, J., et al., *Cell* 57:469-481 (1989)] were kindly provided by Dr. Raymond Sweet (SmithKline Beecham). The soluble VCAM protein used in these studies is a chimera of human D1D2 VCAM

and the murine constant kappa chain. The soluble VCAM was expressed in SF9 cells by a recombinant baculovirus and purified on a Protein A column.

Gp120 Binding Assay

The YU2ΔC1ΔV1/2ΔC5 protein was iodinated to a specific activity of 900 Ci/mmol using solid-phase lactoperoxidase and glucose oxidase (Enzymobeads, BioRad, Richmond, CA) [Gerard, N.P., et al., *J Biol Chem* 264:1760-1766 (1989)]. The CCR5F-L1.2 cells were preincubated for 10-20 minutes at room temperature in phosphate-buffered saline. Then the labeled protein was added (final concentration 0.1 nM to 5 x 10^5 cells in duplicate in 100 µl of 50 mM HEPES, pH 7.2 containing 1mM $CaCl_2$, 5 mM $MgCl_2$ 0.5% BSA, 100 nM sCD4 and different concentrations of unlabeled YU2ΔC1ΔV1/2ΔC5 or HXBc2ΔC1ΔC5 protein. For chemokine inhibition, the iodinated YU2ΔC1ΔV1/2ΔC5 protein was incubated with the CCR5F-L1.2 cells in the same buffer containing 100 nM sCD4, 100 nM HXBc2ΔC1ΔC5 and different concentrations of RANTES MIP-1α, MIP-1β or the HXBc2ΔC1ΔV1/2/3ΔC5 protein. After 30 minutes of incubation at 37° C, cells were washed in 50 mM HEPES, pH 7.2, 1 mM $CaCl_2$, 5 mM $MgCl_2$, 0.5% BSA and 0.5 M NaCl and bound radioactivity counted. The background (non-specific) binding was determined in the presence of 100 nM unlabeled YU2ΔC1ΔV1/2ΔC5 protein. The percent specific binding was calculated using the following formula:

$$\% \text{ Specific Binding} = 100 \times \frac{(S-B)}{(T-B)}$$

where S represents the observed counts bound at a given concentration of unlabeled competitor protein, T represents the observed counts bound in the absence of competitor, and B represents the background counts.

The ability of purified gp120 exterior envelope glycoproteins from macrophage-tropic primary or laboratory-adapted HIV-1 to compete with the natural ligands for CCR5 was studied in the absence and presence of soluble forms of CD4 [Hussey, R., et al., *Nature* 331:78-81 (1988); Arthos, J., et al., *Cell* 57:469-481 (1989)]. Radiolabeled MIP-1α, MIP-1β and RANTES, but not MCP-1 or IL-8, bound to CCR5F-L1.2 cells, which are murine lymphocytes stably expressing an epitope-tagged CCR5 protein (5-8 x 10^4 binding sites/cell), with dissociation constants of 1.1, 0.4 and 0.2 nM, respectively (data not shown). The HIV-1 gp120 derivatives used in this

study (Figure 9A) were derived from the JR-FL, BAL and YU2 macrophage-tropic primary viruses or from the HXBc2 laboratory adapted virus. Some of the gp120 glycoproteins contain deletions of the first (C1) and fifth (C5) conserved regions, which are important for the gp120 interaction with the gp41 transmembrane glycoprotein [Helseth, E., et al., *J. Virol* 65:2119-2123 (1991)], or deletions of the major V1/V2 or V3 variable loops [Wyatt, R., et al., *J Virol* 67:4557-4565 (1993); Wyatt, R., et al., *J Virol* 69:5723-5733 (1995)]. All of the gp120 derivatives used in this study have been shown to bind CD4 efficiently, with dissociation constants of 4-30 nM and to bind the F105 and 17b antibodies in the absence and presence of soluble CD4, respectively [Wyatt, R., et al., *J Virol* 67:4557-4565 (1993); Wyatt, R., et al., *J Virol* 69:5723-5733 (1995); Ivey-Hoyle, M., *Proc Natl Acad Sci USA* 88:512-516 (1991). The F105 antibody recognizes a discontinuous HIV-1 gp120 epitope that overlaps the CD4 binding site [Posner, M., et al., *J Immunol* 146:4325-4332 (1991)], while the 17b antibody binds a discontinuous gp120 epitope that is increased in exposure following CD4 binding [Thali, M., *J Virol* 67:3978-3988 (1993)]. Two soluble forms of the CD4 glycoprotein were included in the study, four-domain soluble CD4 (sCD4) and a protein consisting of the amino-terminal two domains of CD4 (D1D2 sCD4) [Hussey, R., et al., *Nature* 331:78-81 (1988);Arthos, J., et al., *Cell* 57:469-481 (1989)]. In the presence of sCD4, the JR-FL, BAL and YU2ΔC1ΔV1/2ΔC5 envelope glycoproteins, which were derived from the macrophage-tropic primary viruses, significantly inhibited MIP-1α binding to CCR5F-L1.2 cells (Figure 9B). A dose-response curve indicated inhibitory concentrations (IC_{50}) of 4,7,5.5 and 0.7 nM for the JR-FL, BAL and YU2ΔC1ΔV1/2ΔC5 glycoproteins, respectively, in the presence of sCD4 (Figure 9C). In the absence of sCD4, 500 nM concentrations of the JR-FL, BAL and YU2ΔC1ΔV1/2ΔC5 glycoproteins inhibited 32-45 percent of MIP-1α binding to CCR5F-L1.2 cells. For these gp120 derivatives, the presence of sCD4 resulted in a 2- to 3- log increase in the efficiency of the observed inhibition.

In contrast to the above results, the YU2ΔC1ΔVI/2/3ΔC5 glycoprotein, which differs from the YU2ΔC1ΔV1/2ΔC5 glycoprotein by the absence of the gp120 V3 loop, was dramatically reduced in the ability to inhibit MIP-1α binding in the presence and absence of sCD4 (Figure9B and 9C). Thus, the V3 loop appears to be critical for the inhibition of MIP-1α

binding to the CCR5F-L1.2 cells. No significant inhibition of MIP-1α binding was observed for any of the HXBc2 envelope glycoprotein derivatives over that seen with sCD4 alone. The latter inhibition was 12 percent or less and did not significantly increase at higher sCD4 concentrations. The inhibition of MIP-1α binding by the JR-FL, BAL and YU2ΔC1ΔV1/2ΔC5 proteins was specific for CCR5F-L1.2 cells, since no significant inhibition of MIP-1α binding to L1.2 cells expressing CCR1 was observed for these envelope glycoproteins, even when sCD4 was present (Figure 9D). Similarly, no inhibition of MIP-1α binding to THP-1 cells was seen for these gp120 glycoprotein sCd4 mixtures (data not shown). The THP-1 cells used in these experiments specifically bound MIP-1α but did not bind MIP-1β, indicating that CCR5 is not efficiently expressed on the cell surface (L.W., unpublished data). The observed chemokine receptor specificity indicates that interaction with MIP-1α is not the basis for the observed inhibition of binding by the gp120 glycoprotein-CD4 complexes. The JR-FL, BAL and YU2ΔC1ΔV1/2ΔC5 glycoproteins, but not the YU2ΔC1ΔV1/2/3ΔC5 or the HXBc2ΔC1ΔC5 glycoproteins, also inhibited MIP-1β binding to CCR5F-L1.2 cells in the presence of sCD4 (Figure 9E). Together these results indicate that MIP-1α and MIP-1β binding to CCR5-expressing cells can be specifically inhibited by gp120 glycoproteins or some gp120 fragments derived from macrophage-tropic primary HIV-1 isolates, that the efficiency of this inhibition is dramatically increased in the presence of soluble CD4, and that an intact V3 loop appears to be important for the effect.

The two-domain soluble CD4 (D1D2 sCD4) was compared to four-domain sCD4 for the ability to promote the high-affinity interaction with CCR5 when mixed with gp120 glycoproteins from macrophage-tropic primary HIV-1. Mixtures of these gp120 glycoproteins with both D1D2 sCD4 and sCD4 efficiently inhibited MIP-1α and MIP-1β binding to CCR5F-L1.2 cells (Figures 10A and B). In contrast to the sCD4 protein, the D1D2 sCD4 inhibited MIP-1α and MIP-1β binding with an IC_{50} of 20-30 nM in the absence of the gp120 glycoprotein. This inhibitory effect of D1D2 sCD4 exhibited chemokine receptor specificity, since the D1D2 sCD4 did not efficiently inhibit MIP-1α binding to CCR1-L1.2 cells (Figure 10C). Soluble

VCAM, another soluble immunoglobulin family protein, had no significant effect on MIP-1α binding to CCR5F-L1.2 cells (Figure 10A).

We examined the effect of anti-gp120 monoclonal antibodies on the inhibition of MIP-1α binding to CCR5F-L1.2 cells by the JR-FL gp 120 glycoprotein in the presence of sCD4 (Figure 11). All of the antibodies included in this study were previously shown to recognize gp120 in the presence of sCD4 [Wyatt, R., et al., *J Virol* 69:5723-5733 (1995);Thali, M., *J Virol* 67:3978-3988 (1993); Moore, J., et al., *J Virol* 69:122-130 (1995); Gershoni J., et al., *FASEB J* 7:1185-1187 (1993)] and were able to precipitate the JR-FL gp120 glycoprotein (data not shown). The 17b neutralizing antibody, which recognizes a discontinuous, conserved gp120 epitope exposed better after CD4 binding [Thali, M., *J Virol* 67:3978-3988 (1993)], blocked the inhibition of MIP-1α binding by the gp120-sCD4 mixture. Inhibition of MIP-1α binding was also decreased by the addition of CG10 antibody, which recognizes an epitope present only on gp120-CD4 complexes [Gershoni J., et al., *FASEB J* 7:1185-1187 (1993)]. Two neutralizing antibodies, 19b and Loop 2, which recognize the V3 loop of the JR-FL gp120 glycoprotein Moore, J., et al., *J Virol* 69:122-130 (1995)], significantly blocked the effect of the JR-FL gp120-CD4 mixture on MIP-1α binding. In contrast to the above results, nonneutralizing antibodies (A32, C11, 2.3A) that recognize gp120 domains interacting with the gp41 transmembrane glycoprotein [Moore, J., et al., *J Virol* 70:1863-1872 (1996)] did not block the ability of gp120-sCD4 complexes to inhibit MIP-1α binding. Of the anti-CD4 antibodies tested (5A8, Q425, R3-47 and L71) [Burkly L., et al., *J Immunol* 149:1779-1787 (1992); Healey, D., et al., *J Exp Med* 172:1233-1242 (1990); Truneh, A., et al., *J Biol Chem* 266:5942-5948 (1991); Bachelder, R., et al., *J Virol* 69:5734-5742 (1995)], only L71, which can decrease gp120-CD4 interaction in some contexts [Truneh, A., et al., *J Biol Chem* 266:5942-5948 (1991)], moderately affected the observed MIP-1α inhibition.

The direct binding of a radiolabeled macrophage-tropic virus gp120 protein YU2ΔC1ΔV1/2ΔC5, to CCR5F-L1.2 cells was examined in the presence of sCD4. The specific binding of the labeled YU2ΔC1ΔV1/2ΔC5 protein to CCR5F-L1.2 cells was efficiently inhibited by the YU2ΔC1ΔV1/2ΔC5 protein but not by the HXBc2ΔC1ΔC5 protein (Figure

12A). Scatchard analysis revealed that the YU2ΔC1ΔV12ΔC5 binding occurred with a dissociation constant of 4-6 nM and that the number of binding sites on the CCR5F-L1.2 cells was similar for the YU2ΔC1ΔV1/2ΔC5 protein and for MIP-1β (data not shown). RANTES, M1P-1α and M1P-1β decreased the binding of the YU2ΔC1ΔV1/2ΔC5 protein to CCR5F-L1.2 cells, whereas the V3 loop-deleted protein, YU2ΔC1ΔV1/2/3ΔC5, did not (Figure 12B). These results indicate that the natural CCR5 ligands inhibit the binding of a macrophage-tropic HIV-1 gp120-sCD4 complex to CCR5-expressing cells.

This demonstrates that the gp120 glycoproteins derived from macrophage-tropic primary HIV-1 specifically interact with CCR5 and inhibit MIP-1α or MIP-1β binding to transfected cells that express CCR5. The gp120-CCR5 interaction was dramatically enhanced by CD4, indicating that a major consequence of CD4 binding is, in addition to virus attachment to the target cell, promotion of subsequent events like chemokine receptor binding that are important for the membrane fusion process. CD4-mediated induction of CCR5 binding may contribute to the observed enhancement of primary HIV-1 infection by sCD4 [Sullivan, N., et al., *J Virol* 69:4413-4422 (1995)]. A sequential, two-step process for viral attachment and entry allows conserved elements on the viral glycoproteins interacting with chemokine receptors to remain sequestered from antiviral antibodies, until such time as proximity to the target cell membrane is achieved by the virus. The limited accessibility of antibodies to CD4-induced gp120 moieties in the context of the membrane-anchored, oligomeric envelope glycoprotein-CD4 complex may then allow membrane fusion and virus entry to proceed in the face of the humoral immune response.

A low affinity interaction with CCR5 can apparently occur in the absence of CD4 for gp120 variants derived from macrophage-tropic primary HIV-1. These results indicate that a major site for CCR5 interaction is contained on the gp120 glycoprotein. The CCR5-interactive region must be reasonably well-conserved, since CCR5 can be used as a coreceptor by diverse HIV-1 strains as well as by simian immunodeficiency viruses [Choe, H., et al., Cell 85:1135-1148 (1996); Marcon, L., et al. submitted]. While additional studies will be required to define this site absolutely precisely, our data provides general information concerning the CCR5-interactive

region. First, the CCR5-interactive region is preserved on a gp120 fragment lacking the C1, V1/V2 and C5 regions. Second, some HIV-1-neutralizing antibodies that do not interfere with gp120-CD4 binding blocked the interaction of soluble CD4-gp120 complexes with CCR5. One of these antibodies, 17b, recognizes a discontinuous gp120 epitope that is exposed better upon CD4 binding [Thali, M., *J Virol* 67:3978-3988 (1993)], a property shared by the CCR5-interactive region. Other antibodies inhibiting CCR5 interaction, 19b and Loop 2 are directed against the gp120 V3 loop [Moore, J., et al., *J Virol* 69:122-130 (1995)]. The 17b and V3 epitopes *per se* cannot represent the CCR5-interactive region on gp120, since natural primate immunodeficiency viruses that are not recognized by the 17b, 19b and Loop 2 antibodies can utilize CCR5 as a coreceptor [Choe, H., et al., Cell 85:1135-1148 (1996); Marcon, et al., submitted]. However, CCR5-gp120 interaction is likely to involve gp120 structures proximal to these epitopes. Third, deletion of the V3 loop, which can determine HIV-1 tropism and chemokine receptor utilization [Choe, H., et al., Cell 85:1135-1148 (1996); Cheng-Mayer, C., et al., *J Virol* 64:4390-4398 (1990); Chesebro, B., et al., *J Virol* 65:5782-5789 (1991); Hwang, S., et al., *Science* 253:71-74 (1991); Westervelt, P., et al., *J Virol* 66:2577-2582 (1992)], disrupted CCR5 interaction. A component of the chemokine receptor binding site on gp120 may reside in a V3 structure demonstrating moderate variability. Together these observations implicate a discontinuous gp120 structure in the vicinity of the 17b epitope, with a probable contribution from V3 sequences, as the CCR5-interactive moiety on the gp120 glycoprotein. The proximity of the 17b epitope and the V3 loop on the native gp120 glycoprotein has been suggested by previous mutational and antibody competition analyses [Wyatt, R., et al., *J Virol* 69:5723-5733 (1995); Thali, M., *J Virol* 67:3978-3988 (1993); Moore, J., et al., *J Virol* 70:1863-1872 (1996)].

Our results indicate that an interaction between D1D2sCD4 and CCR5 occurs in the absence of gp120 glycoproteins. Since full-length sCD4 did not compete for MIP-1α and MIP-1β binding to CCR5-expressing cells at comparable concentrations, the D1D2 sCD4 appears to more efficiently expose a CCR5-interactive region. That a CD4 fragment spontaneously and specifically binds CCR5 raises the possibility that native CD4 sequences directly contribute to a high affinity binding site for CCR5 in the presence of

the appropriate gp120 glycoprotein. If the observed D1D2 sCD4-CCR5 interaction is biologically relevant, it is probably not restricted to CCR5, since other chemokine receptors as discussed above can be used as coreceptors for HIV-1 variants [Feng, Y., et al., Science 272:872-877 (1996); Choe, H., et al., Cell 85:1135-1148 (1996); Doranze, B.J., et al., *Cell* 85:1149-1158 (1996)]. The regions of gp120 and CD4 that appear to contribute to CCR5 interaction are expected to be quite distant from the target cell membrane, at least upon initial attachment of the virus to CD4. This implies that significant conformational rearrangements of envelope glycoprotein-CD4 complexes may occur during the course of HIV-1 entry to optimize CCR5 binding. Exploiting the interactive regions and mechanisms should facilitate therapeutic and prophylactic strategies targeting HIV-1 entry.

Plasmids

The pHXBH10ΔenvCAT and pSVIIIenv plasmids used to produce recombinant HIV-1 virions have been previously described (Helseth *et al.*, 1990; Thali *et al.*, 1994). The pCD4 plasmid expressing full-length human CD4 has been described (Brand *et al.*, 1995). The SV-A-MLF-Env plasmid expressing the amphotropic murine leukemia virus envelope glycoproteins was obtained from Dan Littman (Landau *et al.*, 1991). The derivation and construction of the pSVIIIenv plasmids expressing the envelope glycoproteins from various strains of HIV-1 have been described (Sullivan *et al.*, 1995; Gao *et al.*, 1996; Karlsson *et al.*, 1996). The chimeric HXBc2 (YU2-V3) and HXBc2 (ADA-V3) *env* constructs were kindly supplied by Lee Ratner, and were designated HY (V3A + V3B) and HA (V3A + V3B) in a previous publication (Carrillo *et al.*, 1993). The chimeric HXBc2 (YU2-V1/V2) *env* genes were created by substituting the Dra III Stu I fragment of the YU2 *env* gene into the corresponding segment (nucleotides 6619 to 6901) of the HXBc2 *env* gene. The cDNAs encoding the chemokine receptors were cloned into the pcDNA3 vector (Invitrogen) for expression. The CCR1, CCR3 and CCR5 proteins, which are known sequences, were also expressed as fusion proteins containing an epitope tag (MDYKDDDDK)(SEQ ID NO:5) (FLAG tag, IBI-Kodak) at the amino terminus.

Cell lines

HeLa cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and antibiotics. HeLa-CD4 (clone 1022) cells were obtained from Dr. Bruce Chesebro through the National Institutes of Health AIDS Research and Reference Reagent Program. The Cf2Th canine thymocyte line was obtained from the American Type Culture Collection (ATCC CRL 1430) and was propagated in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum.

Env-complementation Assay

HeLa cells were cotransfected by the calcium phosphate method (Cullen, 1989) either with 15 μg pHXBH10ΔenvCAT alone or with 15μg pHXBH10ΔenvCAT and 3 μg pSVIIIenv or SV-A-MLV-Env to produce recombinant virions, as previously described (Thali *et al.*, 1994; Karlsson *et al.*, 1996). HeLa cells to be used as target cells were plated at 7 X 10^5 cells per 100 mm dish, cultured overnight, and then transfected by the calcium phosphate method Mg pcDNA3 expressing chemokine receptors. Control HeLa cells were transfected with 10 Mg pCD4 and 25 with 10 μg pCD4 and 25 μg pCDM8 expressing the CD2 protein, which has been shown to have no effect on HIV-1 infection (H.-R. Choe and J. Sodroski, unpublished observations). The pCDM8 plasmid expressing CD2 was a gift from Dr. Ellis Reinherz. Sixty hours following transfection, the HeLa target cells were detached from the tissue culture dish by treatment with phosphate-buffered saline and 5mM EDTA. The cell suspension was diluted in medium, with one aliquot used for FACS analysis and the remaining aliquots replated into 6-well plates for infection. The level of CD4 expressed on the cell surface was measured by flow cytometry, using the FITC-conjugated OKT4 antibody reactive with CD4 domain 3 (McDougal *et al.*, 1986). Approximately six hours after replanting, cells were infected by incubation with recombinant virions (20,000 cpm of reverse transcriptase activity) in 1 ml of medium. After overnight incubation at 37°C, additional medium was added to the cells. After a total of approximately 60 hours of incubation of the virus-cell mixture at 37°C, the cells were lysed and used for determination of CAT activity.

For some of the experiments, the level of chemokine receptor expression on the transfected HeLa cells was measured by FACS analysis 60 hours following transfection. The antibody (anti-FLAG M2, Kodak) against

the epitope tag (FLAG tag) was used for analysis of CCR1, CCR3 and CCR5 expression. Monoclonal antibodies were used to detect surface expression of CCR2, Il8R-A and IL8R-B, respectively.

In some of the experiments, Cf2Th canine thymocytes were used as target cells. The Cf2Th cells were transfected by the calcium phosphate technique with 10 μg of the pCD4 plasmid and 25 μg of the pcDNA3 plasmid expressing chemokine receptors or, as a control, with 10 μg of the pCD4 plasmid and 25 μg of the pCDM8 plasmid expressing CD2 (see above). Approximately 72 hours after transfection, the Cf2Th cells were incubated with recombinant HIV-1 and used for measurement of CAT as described above.

Eotaxin Inhibition of HIV-1 Infectivity

Recombinant HIV-1 containing the YU2 and A-MuLV envelope glycoproteins were produced in HeLa cells as described above. HeLa-CD4 (clone 1022) cells, transfected either with the pCDM8 plasmid expressing CD2 or with the pCDNA3 plasmid expressing chemokine receptors, were used as target cells. The target cells, in 1 ml medium, were incubated with different concentrations (0-60 nM) of eotaxin (Jose et al., 1994; Ponath et al., 1996a and which is commercially available) for 90 minutes at 37°C. Medium was then removed and the cells were resuspended in 1 ml medium containing recombinant virus (15,000 reverse transcriptase units). Eotaxin was added to the virus-cell mixture at the original concentration. After 12 hours at 37°C, the cells were washed and returned to the incubator. After an additional 60 hours at 37°C, the cells were lysed and used for measurement of CAT activity.

Syncytium Formation Assay

Envelope glycoprotein-expressing HeLa cells were derived by transfection of HeLa cells with psVIIIenv plasmids expressing HIV-1 envelope glycoproteins (Helseth *et al.*, 1990). Target cells were derived by transfection of HeLa-CD4 (clone 1022) cells with plasmids expressing either CCR1,CCR3 or CCR5. Forty-eight hours after transfection, the envelope glycoprotein expressing and target HeLa cells were detached from the tissue culture plates using 5 mM EDTA. Cells were replated at a ratio of ten target cells to one envelope glycoprotein-expressing cell, and incubated at 37°C in 5% CO_2. Twelve hours later, the number of syncytia in the wells were

counted. Control experiments were performed in which 2 μg/ml OKT4a (Ortho Pharmaceuticals, Inc.) was included at the time of replating. An additional control using the pCEP4 plasmid (Invitrogen), which does not express any envelope glycoproteins, was performed to assess background levels of syncytia.

As shown in Table 1, the expression of most of the seven-transmembrane receptors did not affect infection by the recombinant HIV-1 viruses. Expression of the CCR5 molecule resulted in significant enhancement of infection by viruses with the ADA, YU2 and Br20-4 envelope glycoproteins, but had no effect on infection by the virus containing the HXBc2 envelope glycoproteins (Figure 1 and Table 1). Expression of the CCR3 molecule also resulted in enhanced infection by the viruses with ADA and YU2 envelope glycoproteins. The magnitude of this effect (9-32-fold) was smaller than that seen for CCR5 (35-45 fold). CCR3 did not stimulate infection by the viruses with BR20-4 and HXBc2 envelope glycoproteins. The enhancing effects of CCR3 and CCR5 expression were not seen when human CD4 was not expressed in the HeLa target cells (Table 1).

The results, shown in Table 2, indicate that CCR5 was able to enhance the infection of a broader array of viruses than was CCR3. The infection of all of the primary viruses than was CCR3. The infection of all the primary viruses was increased in cells expressing CD4 and CCR5 relative to that seen in cells expressing CD4 and CD2 or CD4 and CCR1F. Of the panel of viruses tested, only those containing the ADA and YU2 envelope glycoproteins infected HeLa cells expressing CD4 and CCR3 more efficiently than HeLa cells expressing CD4 and CD2. Recombinant viruses containing laboratory-adapted (HXBc2) viral envelope glycoproteins did not infect HeLa cells expressing either CCR3 or CCR5 more efficiently than they infected control cells expressing CD4 and CD2 or CD4 and CCR1F. Infection by control HIV-1 viruses pseudotyped with the amphotropic murine leukemia virus (A-MuLV) envelope glycoproteins (Landau *et al.*, 1991) was not increased by the expression of CCR3 and CCR5 on the target cells.

The data in Figure 2 indicate that eotaxin exhibited a dose-dependent inhibition of infection of HeLa-CD4 cells expressing CCR3 by YU2

recombinant viruses. No effect of eotaxin was observed, even at high concentrations, on infection by the recombinant viruses with the A-MuLV envelope glycoproteins. No effect of eotaxin was observed on the infection of CCR5-expressing HeLa-CD4 cells by the YU2 recombinant virus. These results indicate that, under circumstances where HIV-1 infection is dependent upon CCR3, eotaxin can inhibit the efficiency of this process.

The results in Figure 3 indicate that none of the recombinant HIV-1 viruses containing the macrophage-tropic primary envelope glycoproteins (ADA, YU2), T cell line-tropic primary envelope glycoproteins (89.6, ELI), or the laboratory-adapted (HXBc2) envelope glycoproteins efficiently infected Cf2Th cells expressing human CD4. Recombinant viruses containing the A-MuLV envelope glycoproteins were able to infect the Cf2Th cells at a high level of efficiency. This was expected since all of the Cf2Th cells in the culture were potentially susceptible to infection by the viruses with A-MuLV envelope glycoproteins. By contrast, only the fraction of cells successfully transfected were potentially infectible by the viruses with HIV-1 envelope glycoproteins. Expression of HUMSTSR in addition to CD4 facilitated infection by the HXBc2 and 89.6 recombinant viruses but did not affect infection by viruses with ADA or YU2 envelope glycoproteins. A small positive effect of HUMSTSR expression was seen on infection by the ELI recombinant virus. These results are consistent with a published report indicating that HUMSTSR expression facilitated cell fusion directed by the envelope glycoproteins of laboratory-adapted HIV-1 but not of macrophage-tropic primary HIV-1 isolates (Feng *et al.*, 1996). The results also demonstrate that HUMSTSR can be utilized by at least some T cell line-tropic primary envelope glycoproteins to facilitate infection. Coexpression of CCR3 with human CD4 enhanced infection by the ADA and YU2

ERROR: ioerror
OFFENDING COMMAND: image

STACK:

-filestream-
-filestream-
-mark-
-savelevel-
-savelevel-